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INTRODUCTION

Breast Cancer is the most common form of cancer in woman in the United States. In 1998, 178,700 new cases of breast cancer were diagnosed, and 43,500 women died from this disease. At a world-wide level breast cancers afflict over 900,000 women per year. It appears that the majority of breast cancers result from a combination of reproductive, environmental (including dietary), lifestyle, and genetic susceptibility factors, but in many cases the precise factors remain to be identified. Also, like other types of cancer, the development of breast cancer is a multistage process that involves the progressive acquisition of mutations and/or aberrant expression of several genes. The genes involved can be highly diverse with respect to their cellular functions. When cells divide and multiply they go through a clock-like mechanism called the cell cycle. One of the genes that plays a critical role in controlling the cell cycle is called *cyclin D1*. In previous studies our laboratory and other investigators found that there is frequently an abnormal increase in the expression of this gene in breast cancer. In recent studies we discovered a new gene called *dip1* (Ref 1). This gene encodes a novel 45kDa nuclear protein that contains a HLH domain characteristic of certain transcription factors (Fig. 1). The overall purpose of this project is to determine whether the *dip1* gene plays a critical role in the development of breast cancer. The results obtained could suggest novel strategies for breast cancer prevention and therapy.

BODY

Hypothesis/Purpose

The overall hypothesis to be tested in this proposal is that DIP1, a novel bHLH protein recently identified in our laboratory, plays a critical role in the development of human breast cancer. If this hypothesis proves to be correct, then DIP1 or proteins that interact with DIP could provide novel targets for breast cancer chemoprevention and/or therapy.

Research accomplished on specific tasks, during year one.

1. Task 1. Determine whether the DIP1 gene is amplified, rearranged and/or altered in its level of expression in human breast cancer cells.

Proteins were extracted from several human breast cancer cell lines and from 20 frozen tissue samples of primary breast cancers. Using an antibody to DIP1 generated in our laboratory we then examined the expression of the DIP1 protein by the technique of Western blot analyses. We found that all of the breast cancer samples expressed fairly similar levels of the DIP1 protein. It is of interest that the normal human mammary epithelial cell line MCF-10F expressed higher levels of DIP1 than the MCF-7 cells. These findings are consistent with our hypothesis that this protein may play an important role in breast cancer.

Southern blot analyses of the DNA extracted from these same breast cancer samples did not reveal any evidence of amplification or gross rearrangements of the *dip1* gene (data not shown).

2. Task 2. Determine the biologic effects of DIP1 by developing derivatives of MCF-7 cells with altered levels of expression of this protein.

The DIP1 cDNA was inserted in the "sense" or anti-sense" orientation into the expression vector pCEP4. We also used a DIP1 cDNA containing a point mutation at residue 28 (Leucine⇒Alanine). This mutant is designated "Dipmut." These vectors were then used to transfect MCF-7 cells and clones isolated from the cells transfected with the "sense" DIP1 or DIP1mut constructs, that stably expressed increased levels of the DIP1 protein when examined by western blot analysis. We were not, however, able to obtain derivatives of the MCF-7 cells transfected with the antisense constructs that expressed decreased levels of the DIP1 protein.

Cell proliferation studies (MTT) assays indicated that the increased expression of DIP inhibited the growth of MCF-7 cells. Inhibition was also observed with the DIP mut construct although this was somewhat less than that obtained with the wild type protein. Representative studies, employing as a control a derivative that was transfected with only the empty pCEP4 vector, are shown in Figure 2.

To exclude the possibility that these results reflected spontaneous clonal variation or secondary changes during selection of the derivatives, we also did transient transfection assays to determine the effects of the DIP1 and DIP1mut constructs on the colony forming ability of MCF-7 cells. The stained plates are shown in Figure 3 and the quantitative results summarized in Figure 4. Both the DIP1 and DIP1mut constructs markedly inhibited colony formation and again, the effect was greatest with DIP1. Thus, DIP caused about 94% inhibition and DIP1mut about 84% inhibition of colony formation (Figure 4).

These studies establish DIP1 as a protein that inhibits rather than stimulates the growth of breast cancer cells, which is consistent with the above mentioned finding that the level of expression of DIP1 in MCF-7 cells is lower than in the normal human mammary epithelial cell line MCF-10F. These findings, do not, however, indicate the mechanism of action of this protein, which is the goal of the studies described below.

Task 3. Studies on the mechanism of action of DIP1, emphasizing its role in gene transcription.

We originally identified DIP1 by its ability to bind to cyclin D1 in the yeast two-hybrid system (1). However, we have not been able to consistently demonstrate, by co-immunoprecipitation, that it binds to cyclin D1 in breast cancer cells. Therefore, we have pursued other directions to elucidate its mechanism of action, focusing on possible effects on gene transcription since DIP1 contains a HLH domain frequently found in transcriptional factors (2-7).

To assess possible effects on gene transcription we did transient transfection-reporter assays, with various promoter elements linked to a luciferase reporter. Transfection assays were done in the absence or presence of the pCEP4 vector itself, the DIP or the DIP1mut vectors. The results indicated that both DIP1 and DIP1mut markedly inhibited both the cyclin D1- and c-fos-luciferase reporters. On the other hand, only slight inhibition by DIP or DIP mut was seen with the SRE-luciferase or CMV-beta gal reporters (Figure 5). The latter result suggests that DIP inhibits the transcription of specific genes and is not simply a non-specific inhibitor of transcription. DIP1 also inhibited the transcription of an estrogen response element (ERE)-luciferase reporter, but this effect was quite variable (data now shown).

Currently there is considerable interest in the role of histone acetylation in stimulating the transcription of specific genes (8-10). The extent of histone acetylation reflects a dynamic balance between the extent of histone acetylation, (for example, on K9, K14 of histone H3) residues by a series of histone acetylation (HATs) enzymes versus the extent of histone deacetylation by a set of histone deacetylases (HDACS) (for review see 11,12). Therefore, we repeated the above studies with the cyclin D1 promoter- and c-fos promoter-luciferase reporters, in the absence and presence of trichostatin A (TSA), an inhibitor of HDAC (13,14). Figure 6 indicates that when added at 50-200 nM TSA caused a dose dependent stimulation of both *c-fos* and cyclin D promoter transcription. In both cases this stimulation was markedly inhibited by DIP1. Indeed, the extent of inhibition by DIP1 was even greater than that obtained in studies done in the absence of TSA (compare Figures 5 and 6).

The above results suggested that DIP1 might interact with a component of transcriptional complexes that affects the state of histone acetylation. Therefore, we examined the possibility that DIP1 binds to one or more HDACs, thereby possibly enhancing their activity. MCF-7 cells were transiently transfected with DIP1, flag-tagged HDAC1 or flag-tagged HDAC3 plasmids. Total protein extracts were prepared 24 hours later and immunoprecipitated with an anti-flag antibody (to pull down HDAC). The immunoprecipitates were then examined by western blot analysis using a DIP1 antibody. In a reciprocal study cos7 cells were transiently transfected with HA-tagged DIP1, HA-tagged Dipmut or flag-tagged HDAC1. Total protein extracts were prepared 24 hours later and immunoprecipitated with a HA antibody (to pull down DIP1) and the immunoprecipitates then examined by western blot analysis with the anti-flag antibody, to detect HDAC1. The results of both studies are shown in Figure 7. They indicate that DIP1 binds to both HDAC1 and HDAC3, and that Dipmut also binds to HDAC1.

4. Discussion/Relevance

The results we have obtained during the first year of this project provide strong evidence that the novel protein DIP1 acts by inhibiting the transcription of specific genes and that this appears to involve, at least in part, effects on the process of histone acetylation. The precise mechanisms of action of DIP1, however, remains to be determined, and this is the major goal of our studies for the coming year. Presumably, this inhibitory effect on transcription explains why we found that overexpression of DIP1 inhibits the growth of MCF-7 human breast cancer cells (Figures 2-4). We are hopeful, therefore, that our further studies on DIP1 will provide new insights into how to more effectively inhibit the development and growth of breast cancers, and thus have clinical relevance.

Future Studies

- a. We will determine whether overexpression of DIP1 in MCF-7 cells has specific effects on cell cycle progression (using flow cytometry), the expression of endogenous cyclin D1, and apoptosis (both spontaneous and drug-induced).
- b. We will construct a series of truncated and mutant forms of DIP1 to map the specific regions of the molecule that play a role in: 1) inhibiting the cyclin D1 promoter- and *c-fos* promoter-luciferase assays, and 2) binding to HDACs.
- c. We will determine, using co-immunoprecipitation studies, whether DIP1 or mutant forms of DIP1 bind to the histone acetylators (HAT) proteins P300/CBP and PCAF. We will also determine the effects of DIP1 on cyclin D1 promoter-luciferase activity in assays done in the presence of HATs instead of TSA.
- d. We will see whether the inhibitory effects of DIP1 on TSA stimulated transcription (Figure 6) are also seen when transcription is stimulated by sodium butyrate or other inhibitors of HDAC.

e. We will use electrophoretic mobility shift assays to determine whether DIP1 or mutants of DIP1 directly inhibit the *in vitro* binding of cellular proteins to a ³²P-labelled oligonucleotide fragment of the cyclin D1 or c-*fos* promoter.

f. We will determine whether DIP1 affects histone acetylation by actually increasing the binding of HDAC to the cyclin D1 promoter, using the chromatin immunoprecipitation (CHIP) method (15,16) and extracts of cells that overexpress DIP1.

6. Figures 1-7 (Attached)

KEY RESEARCH ACCOMPLISHMENTS

1. We have found that DIP1, a novel 45 Kda nuclear protein recently identified in our laboratory, is frequently expressed in human breast cancer.

2. Overexpression of this protein in MCF-7 human breast cancer cells markedly inhibits growth.

3. Mechanistic studies provide evidence that DIP1 inhibits the transcription of specific genes by affecting the process of histone acetylation.

REPORTABLE OUTCOMES

Manuscripts/Abstracts

1. Yao Yao, Yuichiro Doke, Wei Jiang, Masays Imoto, V.S.Venkatraj, Dorothy Warburon, Regina M. Santella Binfeng Lu, Llunbiao Yan, Xiao-Hong Sum Tao Su, Jingqing Luo and I. Bernard Weinstein, Cloning and characterization of DIP1, a novel protein that is related to the Id family of proteins. *Exptl. Cell Research*, **257**, 22-32, 2000.
2. Tao Su, J.Q. Luo, Y. Yao and I Bernard Weinstein, Relevance of DIP1, a novel cyclin D1 binding protein to breast cancer. Annual Meeting of the American Association for Cancer Research, San Francisco, CA., April 2000.

Development of cell lines:

Derivatives of MCF-7 that stably overexpress DIP1

Training:

During the course of these studies Dr. Tao Su, a post-doctoral research scientist, obtained extensive training in breast cancer research and in gene transcription.

CONCLUSIONS

A novel nuclear protein designated DIP1 has been identified that inhibits the growth and the transcription of specific genes in human breast cancer cells. These results might suggest new approaches to breast cancer prevention and therapy.

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1   GTTGTCTGTCGGTGGAGCGGCTGTGCGCAGTGC GGCTCCGGCAGTGGCAGCGGAGGCTGTGTTTGC GGCCTTCGGCAAGC
80  GACTGAG ATG GCG AGC GCA ACT GCA CCT GCA GCC GCA GTC CCC ACC CTG GCT TCG CCT TTG
1   M   A   S   A   T   A   P   A   A   A   V   P   T   L   A   S   P   L
142 GAG CAG CTC CGG CAC TTG GCG GAG GAG CTG CGG TTG CTC CTG CCT CGA GTG CGG GTC GGC
19  E   Q   L   R   H   L   A   E   E   L   R   L   L   L   P   R   V   R   V   G
202 GAA GCC CAG GAG ACC ACC GAG GAG TTT AAT CGA GAG ATG TTC TGG AGA AGA CTC AAT GAG
39  E   A   Q   E   T   T   E   E   F   N   R   E   M   F   W   R   R   L   N   E
262 GCA GCT GTG ACT GTG TCA AGG GAA GCC ACG ACT CTG ACC ATA GTC TTC TCT CAG CTT CCA
59  A   A   V   T   V   S   R   E   A   T   T   L   T   I   V   F   S   Q   L   P
322 CTG CCG TCT CCA CAG GAA ACC CAG AAG TTC TGT GAA CAA GTC CAT GCT GCC ATC AAG GCA
79  L   P   S   P   Q   E   T   Q   K   F   C   E   Q   V   H   A   A   I   K   A
382 TTT ATT GCA GTG TAC TAT TTG CTT CCA AAG GAT CAG GGG ATC ACC CTG AGA AAG CTG GTA
99  F   I   A   V   Y   Y   L   L   P   K   D   Q   G   I   T   L   R   K   L   V
442 CGG GGC GCC ACC CTG GAC ATC GTG GAT GGC ATG GCT CAG CTC ATG GAA GTA CTT TCC GTC
119 R   G   A   T   L   D   I   V   D   G   M   A   Q   L   M   E   V   L   S   V
502 ACT CCA ACT CAG AGC CCT GAG AAC AAT GAC CTT ATT TCC TAC AAC AGT GTC TGG GTT GCG
139 T   P   T   Q   S   P   E   N   N   D   L   I   S   Y   N   S   V   W   V   A
562 TGC CAG CAG ATG CCT CAG ATA CCA AGA GAT AAC AAA GCT GCA GCT CTT TTG ATG CTG ACC
159 C   O   Q   M   P   Q   I   P   R   D   N   K   A   A   A   L   L   M   L   T
622 AAG AAT GTG GAT TTT GTG AAG GAT GCA CAT GAA GAA ATG GAG CAG GCT GTG GAA GAA TGT
179 K   N   V   D   F   V   K   D   A   H   E   E   M   E   Q   A   V   E   E   C
682 GAC CCT TAC TCT GGC CTC TTG AAT GAT ACT GAG GAG AAC AAC TCT GAC AAC CAC AAT CAT
199 D   P   Y   S   G   L   L   N   D   T   E   E   N   N   S   D   N   H   N   H
742 GAG GAT GAT GTG TTG GGG TTT CCC AGC AAT CAG GAC TTG TAT TGG TCA GAG GAC GAT CAA
219 E   D   D   V   L   G   F   P   S   N   Q   D   L   Y   W   S   E   D   D   Q
802 GAG CTC ATA ATC CCA TGC CTT GCG CTG GTG AGA GCA TCC AAA GCC TGC CTG AAG AAA ATT
239 E   L   I   I   I   P   C   L   A   L   V   R   A   S   K   A   C   L   K   K   I
862 CGG ATG TTA GTG GCA GAG AAT GGG AAG AAG GAT CAG GTG GCA CAG CTG GAT GAC ATT GTG
259 R   M   L   V   A   E   N   G   K   K   D   Q   V   A   Q   L   D   D   I   V
922 GAT ATT TCT GAT GAA ATC AGC CCT AGT GTG GAT GAT TTG GCT CTG AGC ATA TAT CCA CCT
279 D   I   S   D   E   I   S   P   S   V   D   D   L   A   L   S   I   Y   P   P
982 ATG TGT CAC CTG ACC GTG CGA ATC AAT TCT GCG AAA CTT GTA TCT GTT TTA AAG AAG GCA
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359 E   L   ...
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1317 TGGTTAGGCCAGATTGACACCTATTTATAAACCATATGCGTATATTTTCTGTGCTATATATGAAAAATAATTGCATGA
1396 TTTCTCATTCCTGAGTCATTTCTCAGAGATTCTAGGAAAGCTGCCTTATTCTCTTTTGCAGTAAAGTATGTTGTTTT
1475 CATTTGAAAGATGTTGATGGTCTCAATAAAATGCTAACTTGCCAGTGATTAAAAA

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Figure 1: The nucleotide sequence of Dip1 cDNA and its deduced amino acid sequence. The cDNA contains a coding region of 1080bp, encoding a protein of 360 amino acids. The protein contains a putative leucine zipper (boldface and underlined), a HLH motif (underlined), a LXXLL motif (double underlined), and an acidic region (open box). The 5' end of the original partial cDNA obtained from the yeast two-hybrid screen is indicated by an arrow. A mutant was constructed in which the leucine residue at position 28 in the LXXLL motif was replaced by alanine. This mutant is designated "Dipmut".

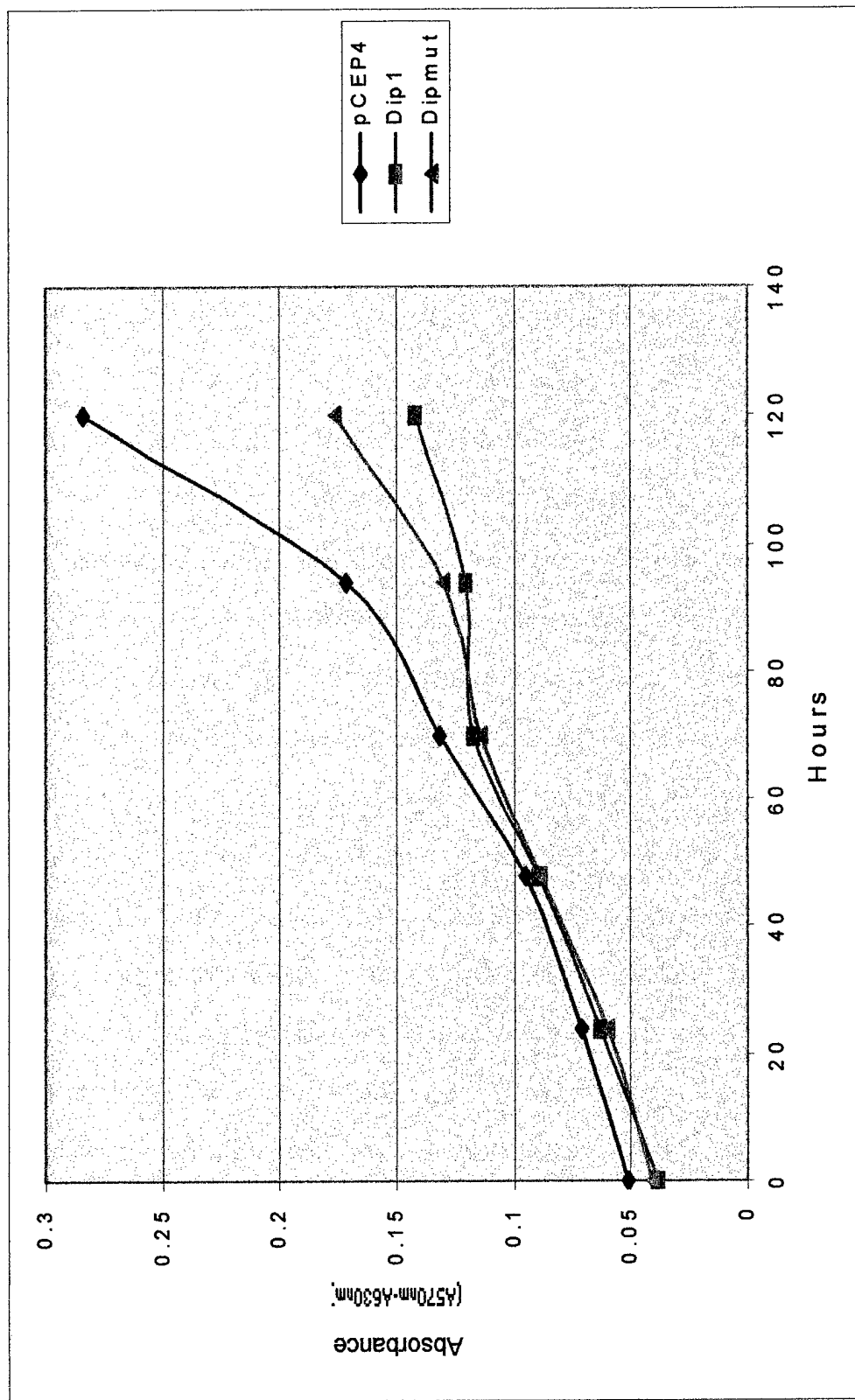


Figure 2: Cell proliferation assays (MTT assays) of MCF7 derivatives that overexpress Dip1. MCF7 cells stably transfected with the pCEP4 vector alone, Dip1 or Dipmut cDNAs were plated individually (1×10^3 cells/well in 96-well plates) and assayed for growth every 24 hours. Overexpression of Dip1 markedly inhibited growth. The Dipmut also inhibited growth but to a lesser extent.

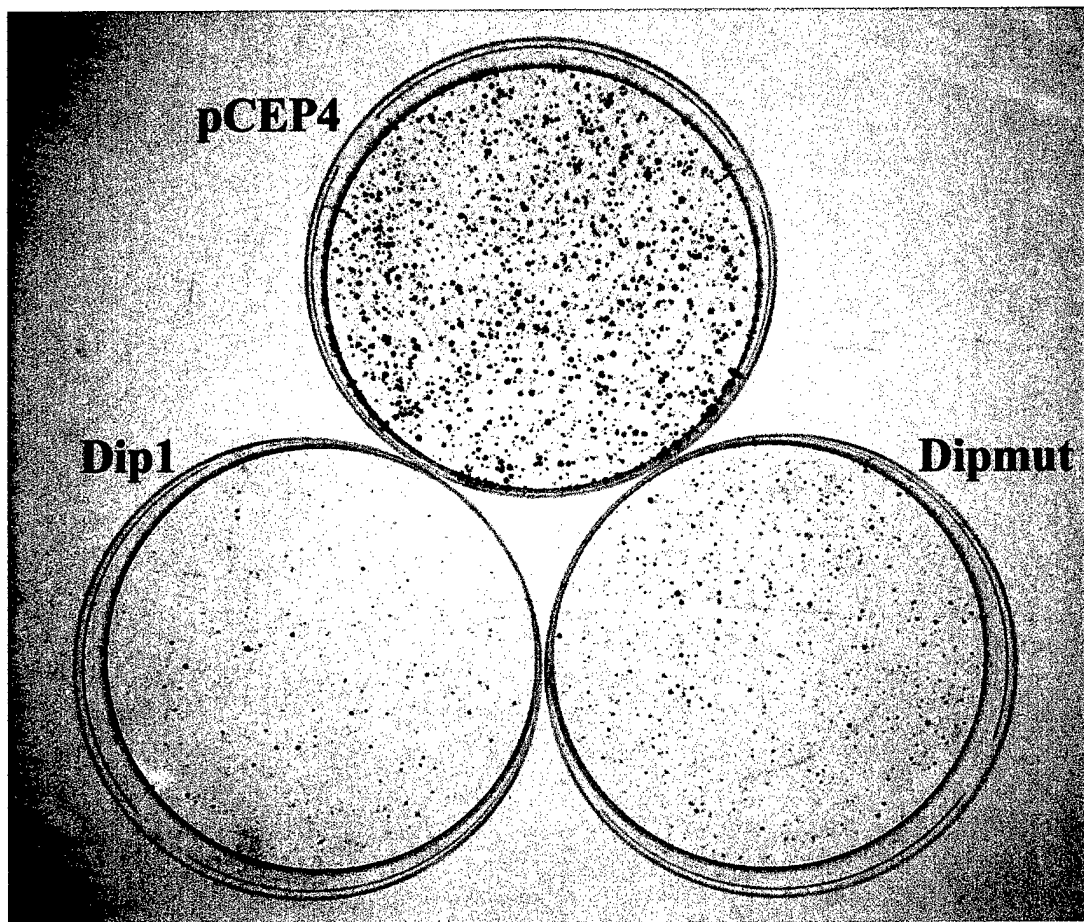


Figure 3: Colony forming assay of MCF7 cells that over-express Dip1. MCF7 cells were plated (1×10^5 cell/10cm dish) and then transfected with either the pCEP4 vector or the vector containing Dip1 or Dipmut cDNA. The cells were then grown in selection medium containing hygromycin for two weeks and then fixed with 37% formaldehyde and stained with 5% Giemsa. Both Dip1 and Dipmut markedly inhibited colony formation. The effect was strongest with Dip1. The quantitative data are shown in Figure 4.

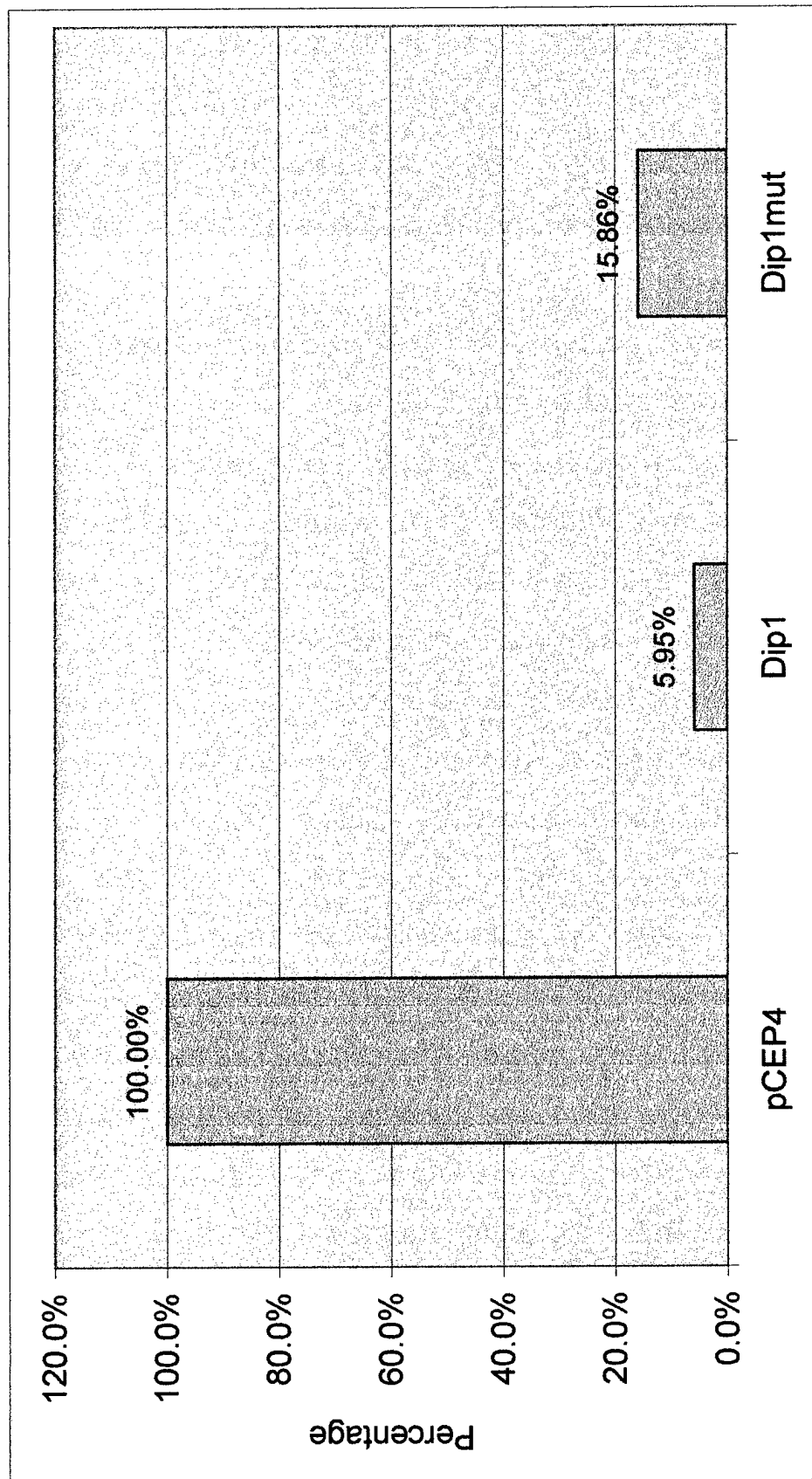


Figure 4: Colony forming assay of MCF7 cells that over-express Dip1. Quantitative data of Figure 3.

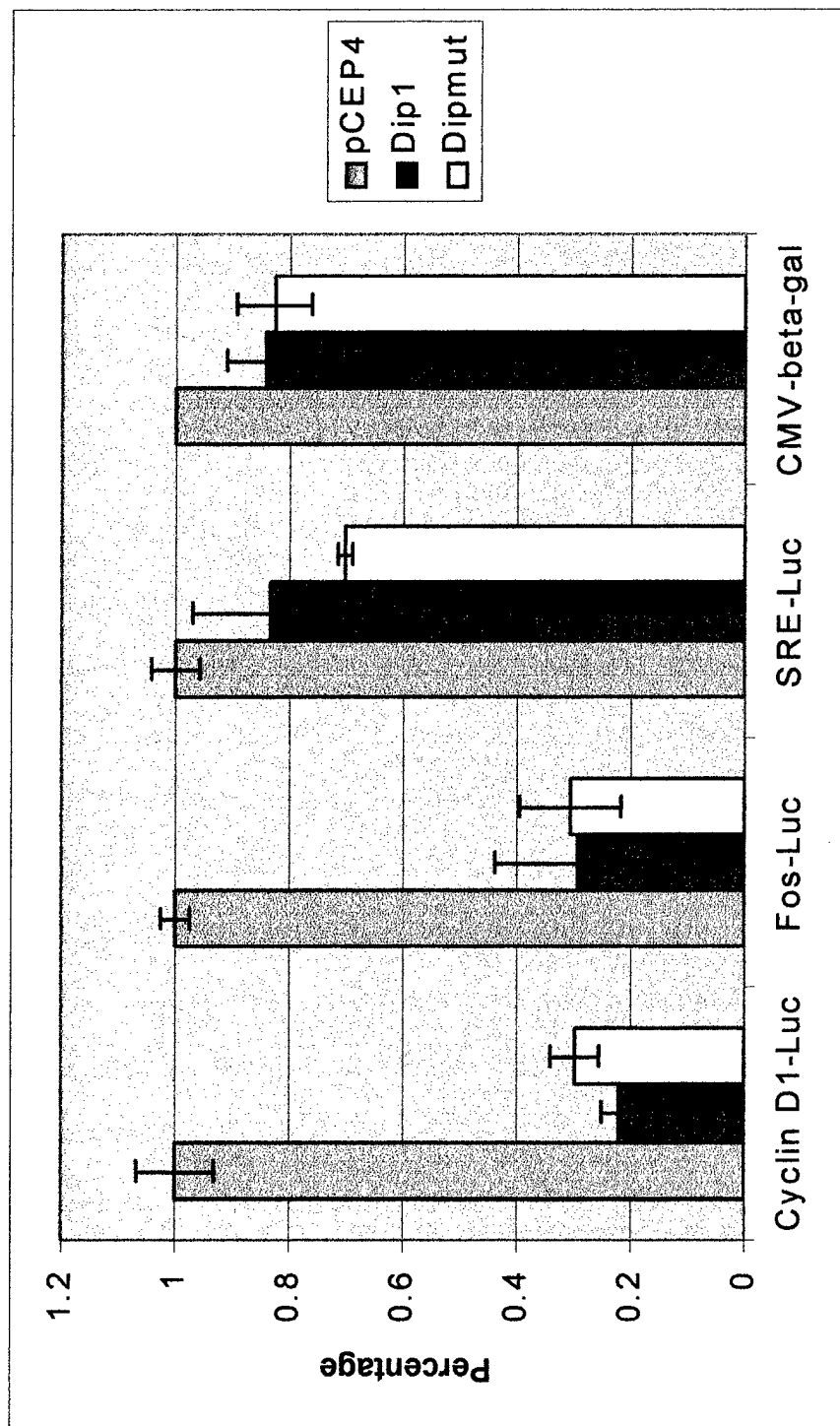


Figure 5: Effects of Dip1 on promoter-luciferase reporter assays in MCF7 cells. MCF7 cells were plated (2×10^5 cells/well in 6-well plates) and then transiently transfected with the pCEP4 vector, Dip1 or Dipmut plasmid DNAs, together with the indicated reporters. Cell extracts were assayed for luciferase or beta-galactosidase 24 hours later. Dip1 and Dipmut markedly inhibited both cyclin D1 and c-fos promoters but only slightly inhibited the serum response element (SRE) and CMV promoters. Dip1 also inhibited the transcription of an estrogen response element (ERE) luciferase reporter but this effect was quite variable (data not shown).

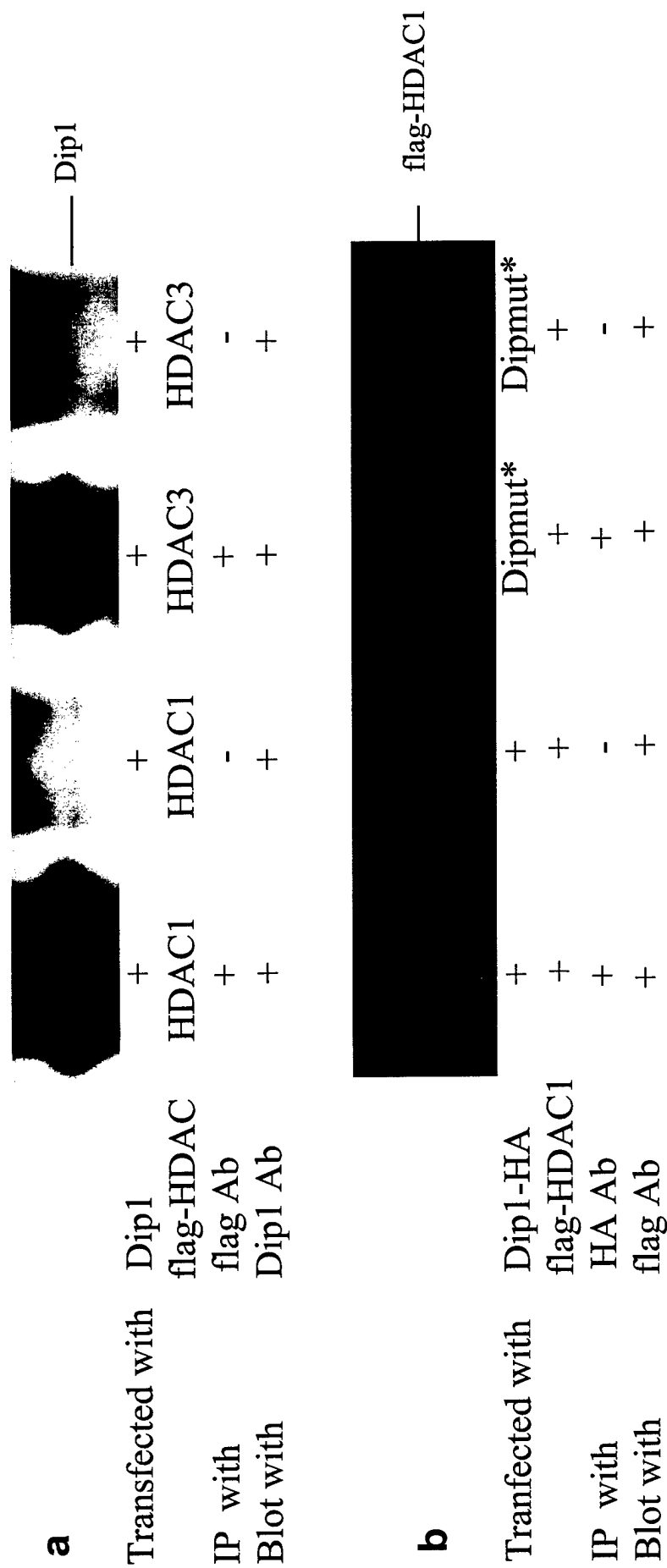


Figure 7: Co-immunoprecipitation of Dip1 and histone deacetylase (HDAC). **a.** MCF7 cells were transiently transfected with Dip1, flag-tagged HDAC1 or flag-tagged HDAC3 plasmids, as indicated. Total protein extracts were prepared 24 hours later and immunoprecipitated with an anti-flag antibody (to pull down HDAC), as indicated. The immunoprecipitates were then analyzed by western blot using a Dip1 antibody. **b.** In a reciprocal study cos7 cells were transiently transfected with HA-tagged Dip1, HA-tagged Dipmut and flag-tagged HDAC1, as indicated. Total protein extracts were prepared 24 hours later and immunoprecipitated with a HA antibody (to pull down Dip1) and the immunoprecipitates then analyzed by western blot with a flag antibody to detect HDAC1. These results indicated that Dip1 binds to both HDAC1 and HDAC3, and that Dipmut also binds to HDAC1.

APPENDICES

2. Yao Yao, Yuichiro Doke, Wei Jiang, Masays Imoto, V.S.Venkatraj, Dorothy Warburon, Regina M. Santella Binfeng Lu, Llunbiao Yan, Xiao-Hong Sum Tao Su, Jingqing Luo and I. Bernard Weinstein, Cloning and characterization of DIP1, a novel protein that is related to the Id family of proteins. *Exptl. Cell Research*, **257**, 22-32, 2000.
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Cloning and Characterization of DIP1, a Novel Protein That Is Related to the Id Family of Proteins

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Using human cyclin D1 as the “bait” in a yeast two-hybrid system, together with a HL60 cDNA library, we identified a novel human nuclear protein designated DIP1. This protein is expressed in a variety of cell types, and in fibroblasts its level remains constant throughout the cell cycle. However, the level of this protein increases severalfold during the differentiation of HL60 cells. The DIP1 protein can be phosphorylated *in vitro* by a cellular kinase and this activity reaches its maximum in extracts obtained from cells in the G1 phase of the cell cycle. DIP1 contains a helix-loop-helix motif but lacks an adjacent basic DNA-binding domain, thus resembling the Id family of proteins. The *dip1* gene is located on human chromosome 16p11.2-12, a locus that is amplified in several types of human cancer. These results suggest that DIP1 may be involved in the control of gene expression and differentiation, but its precise function remains to be determined. © 2000 Academic Press

Key Words: DIP1; cyclin D1; Id; HL60; differentiation.

INTRODUCTION

Cell cycle progression in eukaryotic organisms is governed by a series of cyclin/Cdk complexes that act at different phases of the cell cycle. In mammalian cells, phosphorylation of the pRb protein is required for S phase entry. This phosphorylation is initially triggered by cyclin D/Cdk4(6) in mid- to late G1 and then by cyclin E/Cdk2, which acts at late G1 and throughout the S phase [1]. In its hypophosphorylated state, pRb can prevent S phase entry by binding to the transcription factor E2F, thus repressing transcription from

promoters containing E2F-binding sites. However, hyperphosphorylation of pRb prevents its interaction with E2F, enabling the untethered E2F to activate the transcription of the respective target genes required for transition through the S phase [2].

There is accumulating evidence that, in addition to its effects on the pRb/E2F pathway, cyclin D1 can affect cell growth and differentiation through other downstream effectors. For example, cyclin D1 can inhibit muscle gene expression in the presence of the nonhyperphosphorylatable form of pRb [3]. In contrast to cyclins A and E, ectopic expression of cyclin D1 inhibits muscle gene activation by a mutated form of myogenin in which two potential inhibitory Cdk phosphorylation sites are absent [3]. The C-terminal acidic region of cyclin D1 is required for inhibition of myogenic basic region helix-loop-helix (bHLH) regulator activity, whereas an intact N-terminal pRb binding motif is not essential [4]. Furthermore, recent studies indicate that cyclin D1 itself can bind directly to several transcription factors or coactivators, including the ER, v-Myb, DMP1, SCR-1, and TAF_{II}250 [5–11], resulting in stimulation (ER) [5, 6, 11] or inhibition (v-Myb, DMP1, and TAF_{II}250) [7, 8, 10] of the functions of these proteins. These findings indicate that cyclin D1 can play a role in modulating the expression of specific genes through mechanisms distinct from its role in cell cycle control. Therefore, it is of interest to search for additional proteins that might interact with cyclin D1.

The bHLH motif defines a class of transcription factors that is central to the regulation of cell growth and differentiation [12]. The function of this motif is to provide a dimerization interface through the formation of a parallel four-helix bundle, resulting in the juxtaposition of two basic DNA recognition α -helices that control sequence-specific DNA binding [13, 14]. The prototype protein of the bHLH superfamily is the myogenic transcription factor MyoD whose function in transactivation requires the formation of a het-

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erodimer with one of the alternatively spliced e2A gene products (E12 or E47) [15]. The MyoD/E2A dimer is disrupted by Id (inhibitor of differentiation or inhibitor of DNA binding) proteins which constitute an additional category of HLH proteins that contain the HLH motif but lack a basic DNA-binding domain [16]. As a consequence, Id proteins are able to antagonize the DNA-binding and transcriptional activation functions of bHLH transcription factors through heterodimerization with the latter factors [16, 17].

In this paper, we describe the cloning and characterization of a human cDNA encoding a novel and ubiquitously expressed nuclear protein, designated DIP1. The DIP1 protein was initially identified by its ability to interact with cyclin D1 in the yeast two-hybrid system. Its structure resembles the Id proteins and is induced during the differentiation of HL60 cells. The *dip1* gene is located on human chromosome 16p11.2-12, a region frequently amplified in several types of human cancers [18]. Thus far we have not been able to definitively demonstrate that DIP1 binds to cyclin D1 in mammalian cells. Therefore, the precise function of this novel protein remains to be determined.

MATERIALS AND METHODS

Molecular cloning and DNA sequencing. Human cyclin D1 cDNA was amplified by PCR, using the 5'-primer GAATTCGAATTCAG-GAAGAGCCCCAGCC-OH and the 3'-primer CGGTGTCGACCGC-CTGCCTGGCG-OH. This PCR fragment was digested with *EcoRI* and *SalI* and subsequently inserted between the *EcoRI* and *SalI* sites of the pSH₂-1 plasmid, which contains the Lex-A DNA-binding domain [19], to form the plasmid pSH₂-1/D1. This plasmid was used as the bait in the yeast two-hybrid screen, as described below. A ~0.8-kb partial *dip1* cDNA was cloned and was then used as a probe to screen a HeLa λ cDNA library to obtain the full-length cDNA. Inserts from five independent λ phage clones were released by *EcoRI* and *NotI* as single-banded fragments and subcloned into pBluescript KS (-) for sequencing. The size of the full-length *dip1* cDNA was confirmed by comparison to the size of its transcript. To generate a GST-DIP1 fusion protein expression plasmid, the full-length *dip1* cDNA was PCR amplified using the 5'-primer GAATTCGAAT-TCTCGGCAAGCGACTGAGATG-OH and the 3'-primer GAATTC-GAATTCTCATAATTCAAGTTCAGTTC-OH, followed by *EcoRI* digestion and then insertion of this fragment into the *EcoRI* site of pGEX-3X (Pharmacia). To generate pcDNA3/DIP1, the *dip1* cDNA was released from pBluescript KS (-) by digestion with *StuI* and *NdeI*, Klenow filled in, and then inserted into the *EcoRV* site of pcDNA3 (Invitrogen). To generate pcDNA3/DIP1(NT), the *dip1* cDNA was digested with *EcoRI* and *BspHI*, Klenow filled in, and then inserted into the *EcoRV* site of pcDNA3. To generate the C-terminal HA-tagged DIP1 expression plasmid, pcDNA3 was modified to contain a coding sequence for the HA epitope in the polylinker region to form pcDNA3/HAC1 (J.-W. Soh, unpublished data). The DIP1 cDNA was PCR amplified using the 5'-primer CTGAGAATTC-CGTTGCTGTGCGGTGGAGC-OH and the 3'-primer CAAATG-GCTAGCAAGAATTCTAATTCAAGTTCAGTTC-OH and was then digested with *EcoRI* followed by subcloning into the *EcoRI* site of pcDNA3/HAC1. To obtain genomic fragments of the *dip1* gene, a λ phage genomic library containing the human genome was generated as previously described [20] and was probed with a ³²P-labeled *dip1* cDNA.

DNA sequencing was performed using the PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Perkin Elmer) and an automatic DNA sequencer (373 DNA sequencer, Perkin Elmer) as described by the manufacturer.

The yeast two-hybrid screen. The yeast two-hybrid screen was performed using a human HL60 cDNA expression library generated in pGADNOT, as previously described [21]. The yeast strain used was CTY10-5d that contains a *lacZ* gene downstream of a LexA operator [22]. Screening was performed as previously described [22].

Generation of an anti-DIP1 monoclonal antibody, 5C9. One mg of the GST-DIP1 fusion protein or the GST protein itself was prepared in 500 μ l of phosphate-buffered saline (PBS), mixed with 500 μ l of CFA (complete Freund's adjuvant, Sigma F-4258), and vortexed until it formed an emulsion. Ten 8-week-old BALB/c mice were injected subsequently with this emulsion, each with 100 μ l. Two weeks later, a second immunization was performed with incomplete Freund's adjuvant. An ELISA was performed after an additional 2 weeks, in which the harvested mouse serum was tested against GST-DIP1 and GST alone. Cell fusion was performed 1 week later using cells from mice with the highest titer against GST-DIP1. A total of 1×10^7 P3 myeloma cells were mixed with 1×10^6 spleen cells in 0.5 ml of fusion solution A (30% PEG, 55% RPMI 1640 medium with 10% fetal bovine serum and 15% DMSO) and rocked for 1 min. This was followed by adding 0.5 ml of fusion solution B (16.7% PEG, 83% RPMI 1640 medium with 10% fetal bovine serum) and the suspension was rocked for an additional 2-3 min. The cells were then resuspended in 25 ml RPMI 1640 medium plus 10% fetal bovine serum and plated into eight 96-well plates. After several rounds of clonal selection and ELISA, clone 5C9 was obtained and the antibody it produced was isotypized as IgG1 κ (Amersham RPN 29).

Fluorescent immunohistochemistry. The human esophageal cancer cell line HCE7 and the simian kidney Cos7 cell line were transiently transfected with pcDNA3/DIP1-HA and the cells were fixed on slides in 90% ethanol/5% acetate, at room temperature for 30 min. The slides were then washed 2 \times with PBS and blocked with 10% milk/PBS at 4°C for 4 h or overnight. The primary antibodies 5C9 (ascites) and anti-HA (12CA5, Boehringer) or mouse IgG (Sigma) were used at 1:100, 5 μ g/ml, and 1:100, respectively, in 1% milk/PBS. After a 4-h incubation with the primary antibodies at 37°C, the slides were washed with PBS for 10 min, three times, and then overlaid with FITC-mouse IgG (Boehringer) at 1:100 in 1% milk/PBS and kept in the dark at 4°C overnight. After washing with PBS, the slides were counterstained with 1 μ g/ml propidium iodide/PBS at room temperature for 45 s, washed with PBS, and mounted. Confocal fluorescent microscopy was performed using a LSM-410 confocal system attached to a Zeiss microscope (Axiovert-100, Thornwood, NY).

Northern blots. Multiple-tissue RNA blots were purchased from Clontech. For preparation of total RNA from HL60 cells, approximately 5×10^6 cells were collected at each time point and lysed in 8 ml GTC buffer (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0, 0.1 M β -mercaptoethanol, 0.5% sarcosyl). Each lysate was layered onto a cushion of 3.16 ml of 5.7 M CsCl/0.1 M EDTA in a 14 \times 94 mm Ultraclear (Beckman) centrifuge tube. The RNA was centrifuged at 32,000 rpm for 18 h at 20°C in a SW41 swinging bucket rotor (Beckman). The resultant RNA pellets were then rinsed with 95% ethanol and dissolved in 400 μ l of elution buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1% SDS). LiCl was added to each sample to a final concentration of 0.4 M along with 1 ml of ethanol and the RNA precipitated overnight at -20°C. The resulting RNA precipitates were then microcentrifuged for 30 min at 4°C, washed with 70% ethanol, and dried under vacuum. RNA samples were resuspended in sterile H₂O and adjusted to a final concentration of 2.5 μ g/ μ l. RNA samples containing 10 μ g of total RNA (4 μ l) were combined with RNA sample buffer (16 μ l) (50% formamide, 6.18% formaldehyde, 20 mM Mops, 5 mM sodium acetate, and 1 mM EDTA), heated at 75°C for 15 min, resolved in a 1% agarose-6%

formaldehyde gel, and then blotted onto Hybond N nylon membrane (Amersham) in 20× SSC (1×, 0.15 M NaCl and 15 mM sodium citrate). The blots were hybridized to a random primed ³²P-labeled *dip1* cDNA probe, washed three times with 0.5× SSC/0.1% SDS at 65°C for 15 min each time, and then autoradiographed using Kodak Xar-5 film in a cassette with intensifying screens. As a control of equal loading, the same membranes were hybridized to a radiolabeled β -actin or GAPDH cDNA probe.

Cell lines and culture conditions. HCE7 and TE3 are two human esophageal cancer cell lines [23]. HSF8 is a primary human foreskin fibroblast culture (unpublished data). All of these cells were maintained in RPMI 1640/10% fetal bovine serum. Cell lines SW480, MCF-7, MCF-10F, PC-3, LNCaP, HeLa, and Cos7 were obtained from ATCC and maintained in medium suggested by the supplier. The HL60 cell line was provided by Dr. H. L. Malech at the NIH and was maintained in RPMI 1640/15% fetal bovine serum.

Protein preparation and Western blot. For Western blotting assays, protein lysates were prepared in K_iIP buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 2.5 mM EGTA, 1.0 mM DTT, 0.1% Tween 20, 10% glycerol, 1.0 mM PMSF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) and sonicated for three 10-s bursts, followed by centrifugation at 14,000 rpm at 4°C for 20 min. The monoclonal antibody 5C9 was used at a final concentration of 1:500 of the ascites fluid. Anti-actin, anti-cyclin D1, and anti-Cdk4 antibodies were purchased from UBI and anti-pRb was purchased from Medical & Biological Laboratories Co., LTD (MBL). Western blotting was performed as previously described [24].

Cell cycle synchronization, flow cytometry, and induction of differentiation. HSF8 cells at passage three that were 50% confluent were maintained in RPMI1640 without serum for 72 h. Cell cycle progression was then stimulated by adding RPMI 1640 medium plus 10% fetal bovine serum. The cells were then trypsinized at different time points after refeeding, washed with PBS, fixed with 70% cold ethanol, and stained with 0.1 mg/ml propidium iodide (Sigma) with 0.6% NP-40 and 2 mg/ml RNase A (Sigma). Fluorescence data were collected with a Coulter EPICS 750 flow cytometer and analyzed with the EASY 2 software to determine DNA content and the percentage of cells in each phase of the cell cycle. For induction of differentiation, HL60 cells were maintained as exponentially dividing cultures in RPMI/15% fetal bovine serum. For each treatment, 1–2 × 10⁵ cells/ml were exposed to 25 ng/ml TPA (LC Laboratories) and 1.2% DMSO (Sigma). The induction of differentiation was confirmed by assaying CD11 expression using flow cytometry [25].

In vitro phosphorylation of GST-DIP1. Protein lysates were prepared from cells in K_iIP buffer supplemented with 1.0 mM NaF, 0.1 mM Na₃VO₄, 10 mM β -glycerophosphate. Then 200 μ g of each lysate was mixed with 2 μ g of agarose bead-bound GST-DIP1 for 1 h at 4°C. The beads were washed in the same buffer three times and once in the kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂, 1.0 mM DTT, 2.5 mM EGTA, 10 mM β -glycerophosphate, 1.0 mM NaF, and 0.1 mM Na₃VO₄). After removing the excess liquid, kinase reactions were performed in a 50- μ l reaction volume containing 1× kinase buffer supplemented with 20 μ Ci [γ -³²P]ATP, for 30 min at 30°C. Reactions were stopped by adding 10 μ l of SDS sample buffer and boiling for 5 min. The samples were then resolved in a 10% SDS-PAGE, and the gels were dried and autoradiographed.

Fluorescence in situ hybridization (FISH). Two independent human *dip1* genomic clones were obtained by screening a human genomic phage library with a ³²P-labeled *dip1* cDNA, as previously described [20]. The phage DNA from tertiary clones were used as the probes for FISH, as previously described [26], to determine the chromosomal localization of *dip1*. About 25 metaphase spreads prepared from PHA-stimulated normal human lymphocyte cultures were hybridized with each probe and examined for fluorescent signals. Chromosome bands were visualized using an enhanced DAPI image.

RESULTS

Cloning and Sequencing of the Human *dip1* cDNA

We used the human cyclin D1 as a "bait" in a yeast two-hybrid screen for cyclin D1-binding proteins, together with a HL60 cDNA library [21]. We obtained a novel partial sequence encompassing the 3' half of the subsequently cloned entire cDNA. This partial cDNA was then used to probe a λ gt 11 HeLa cDNA library and a 1.5-kb cDNA was obtained, which we designated *dip1* (D-type cyclin-interacting protein 1). This 1.5-kb cDNA contained the entire open reading frame of a novel protein since it was about the same size as its mRNA (see below). Furthermore, the size of the DIP1 protein translated *in vitro* or ectopically expressed in mammalian cells, encoded by this cDNA, was the same as that of the endogenous DIP1 protein (data not shown). Sequence analysis revealed that the *dip1* cDNA encoded a protein of 360 residues with no significant homology to other known proteins in GenBank, except for Maid, a maternal Id-like transcript identified in the mouse embryo [27]. The deduced amino acid sequence of DIP1 showed that it contained a putative leucine zipper at its N-terminus, a HLH domain in the center, and an acidic C-terminal region (Fig. 1). Multiple sequence alignments with other HLH proteins (Fig. 2) confirmed the presence of the HLH domain in DIP1, although the homology within the first helix is relatively low (only 13%). This HLH domain was not adjacent to a basic DNA-binding domain. These structural aspects of DIP1 are similar to those of the Id family of proteins, which act as dominant negative regulators of transcription [15]. Secondary structure predictions using the PHD or COILS program also revealed an α helical region or a coiled-coil domain spanning the region from Lys¹⁷⁰ to Asp²²⁰ of the DIP1 sequence (data not shown). This result is consistent with a previous report that the E2A protein also contains this structural domain at its HLH region [14]. The similarity between DIP1 and Id proteins raised the possibility that, like Id proteins, it might be involved in transcriptional regulation.

Nuclear Localization of the DIP1 Protein

To facilitate further studies we next examined the intracellular distribution of DIP1. Immunofluorescent staining was performed using the 5C9 antibody (Fig. 3). The endogenous DIP1 protein was concentrated in the nucleus of the human esophageal cancer cell line HCE7 (Fig. 3A) and in several other human cell lines and in paraffin-embedded human tissue sections (data not shown). Cytoplasmic staining of DIP1 was also detected, to a lesser extent, in human foreskin fibroblasts HSF8 (data not shown). To exclude the possibility of nonspecific binding of the 5C9 antibody, HA-

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1   GTTGCTGTGCGGTGGAGCGGCTGTGCGAGTGGCGCTCCGGCAGTGGCAGCGGAGGCTGTGTTGCGGCCCTTCGGCAAGC
80  GACTGAG ATG GCG AGC GCA ACT GCA CCT GCA GCC GCA GTC CCC ACC CTG GCT TCG CCT TTG
1   M   A   S   A   T   A   P   A   A   A   V   P   T   L   A   S   P   L
142 GAG CAG CTC CGG CAC TTG GCG GAG GAG CTG CGG TTG CTC CTG CCT CGA GTG CGG GTC GGC
19  E   Q   L   R   H   L   A   E   E   L   R   L   L   L   P   R   V   R   V   G
202 GAA GCC CAG GAG ACC ACC GAG GAG TTT AAT CGA GAG ATG TTC TGG AGA AGA CTC AAT GAG
39  E   A   Q   E   T   T   E   E   F   N   R   E   M   F   W   R   L   R   N   E
262 GCA GCT GTG ACT GTG TCA AGG GAA GCC ACG ACT CTG ACC ATA GTC TTC TCT CAG CTT CCA
59  A   A   V   T   Y   S   R   E   A   T   T   L   T   I   V   F   S   Q   L   P
322 CTG CCG TCT CCA CAG GAA ACC CAG AAG TTC TGT GAA CAA GTC CAT GCT GCC ATC AAG GCA
79  L   P   S   P   Q   E   T   T   Q   K   F   C   E   Q   V   H   A   A   I   K   A
382 TTT ATT GCA GTG TAC TAT TTG CTT CCA AAG GAT CAG GGG ATC ACC CTG AGA AAG CTG GTA
99  F   I   A   V   Y   Y   L   L   P   K   D   Q   G   I   T   L   R   K   L   V
442 CGG GGC GCC ACC CTG GAC ATC GTG GAT GGC ATG GCT CAG CTC ATG GAA GTA CTT TCC GTC
119 R   G   A   T   L   D   I   V   D   G   M   A   Q   L   M   E   V   L   S   V
502 ACT CCA ACT CAG AGC CCT GAG AAC AAT GAC CTT ATT TCC TAC AAC AGT GTC TGG GTT GCG
139 T   P   T   Q   S   P   E   N   N   D   L   I   S   Y   N   S   V   W   V   A
562 TGC CAG CAG ATG CCT CAG ATA CCA AGA GAT AAC AAA GCT GCA GCT CTT TTG ATG CTG ACC
159 C   Q   Q   Q   M   P   T   Q   I   P   R   D   N   K   A   A   A   L   L   M   L   T
622 AAG AAT GTG GAT TTT GTG AAG GAT GCA CAT GAA GAA ATG GAG CAG GCT GTG GAA GAA TGT
179 K   N   V   D   F   V   K   D   A   H   E   E   M   E   Q   A   V   E   E   C
682 GAC CCT TAC TCT GGC CTC TTG AAT GAT ACT GAG GAG AAC AAC TCT GAC AAC CAC AAT CAT
199 D   P   Y   S   G   L   L   N   D   T   E   E   N   N   S   D   N   H   N   H
712 GAG GAT GAT GTG TTG GGG TTT CCC AGC AAT CAG GAC TAT TGG TCA GAG GAC CAA
249 E   D   D   V   L   G   F   P   S   N   Q   D   L   Y   W   S   E   D   D   Q
802 GAG CTC ATA ATC CCA TGC CTT GCG CTG GTG AGA GCA TCC AAA GCC TGC CTG AAG AAA ATT
239 E   L   I   I   P   C   L   A   L   V   R   A   S   K   A   C   L   K   K   I
862 CGG ATG TTA GTG GCA GAG AAT GGG AAG AAG GAT CAG GTG GCA CAG CTG GAT GAC ATT GTG
922 R   M   L   V   A   E   N   G   K   K   D   Q   V   A   Q   L   D   D   I   V
259 GAT ATT TCT GAT GAA ATC AGC CCT AGT GTG GAT GAT TTG GCT CTG AGC ATA TAT CCA CCT
279 D   I   S   D   E   I   S   P   S   V   D   D   L   A   L   S   I   Y   P   P
982 ATG TGT CAC CTG ACC GTG CGA ATC AAT TCT GCG AAA CTT GTA TCT GTT TTA AAG AAG GCA
299 M   C   H   L   T   V   R   I   N   S   A   K   L   V   S   V   L   K   K   A
1042 CTT GAA ATT ACA AAA GCA AGT CAT GTG ACC CCT CAG CCA GAA GAT AGT TGG ATC CCT TTA
319 L   E   I   T   K   A   S   H   V   T   P   Q   P   E   D   S   W   I   P   L
1102 CTT ATT AAT GCC ATT GAT CAT TGC ATG AAT AGA ATC AAG GAG CTC ACT CAG AGT GAA CTT
339 L   I   N   A   I   D   H   C   M   N   R   I   K   E   L   T   Q   S   E   L
1162 GAA TTA TGA CTTTCAGGCTCATTGTACTCTCTCCCTCTCATGTCATGGTCAGGCTCTGATACCTGCTTTTA
359 E   L   ...
1238 AAATGGAGCTAGAATGCTTGCTGGATTGAAAGGGAGTGCCTATCTATATTTAGCAAGAGACACTATTACCAAGATTGT
1317 TGGTTAGGCCAGATTGACACCTATTATATAAACCATATGCGTATATTTTCTGTGCTATATATGAAAAATAATTGCATGA
1396 TTTCTCATTCCTGAGTCATTCTCAGAGATTCCTAGGAAAGCTGCCTTATTCTCTTTTGCAGTAAAGTATGTGTTT
1475 CATTGTAAGATGTTGATGGTCTCAATAAAATGCTAAGTCCAGTGATTAAAAAAGTATGTTGTTT

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FIG. 1. The nucleotide sequence of *dip1* cDNA and its deduced amino acid sequence. The cDNA contains a coding region of 1080 bp, encoding a protein of 360 amino acids. The protein contains a putative leucine zipper (boldface and underlined), a HLH motif (underlined), and an acidic region (open box). The 5' end of the original partial cDNA obtained from the yeast two-hybrid screen is indicated by an arrow. The nuclear polyadenylation site is marked with asterisks. The GenBank accession number for DIP1 cDNA is AF082569.

tagged DIP1 was transiently overexpressed in Cos7 cells and immunofluorescent staining was then carried out with either the 5C9 or the anti-HA (12CA5) antibodies. The exogenous DIP1 was detected mainly in the nucleus with both antibodies (Figs. 3C and 3E), while a mouse preimmune control serum showed negative staining (Fig. 3G).

Ubiquitous Expression of DIP1

As an approach to the function of the DIP1 protein, we first analyzed DIP1 expression patterns at both the mRNA and the protein levels. Northern blot analysis using a *dip1* cDNA probe was performed on multitissue blots containing mRNA from 16 different normal hu-

man tissues (Clontech) (Fig. 4a). All of these tissues expressed abundant levels of a ~1.6-kb DIP1 mRNA, except for the lung and liver samples in which DIP1 expression was relatively low. The significance of a second less abundant and larger size (about 4 kb) mRNA band in some of these tissues is not known. It might be partially processed DIP1 mRNA, since the intensities of these two bands in different tissues are proportional. The use of a β -actin probe confirmed the approximately equal loading of RNA samples in these lanes; the increase in β -actin mRNA in the heart and skeletal muscle samples was expected. A Western blot analysis using protein lysates prepared from representative human cell lines indicated that the DIP1 protein

	basic	helix 1	loop	helix 2
L-Myc	VTKRNHNFLERKRRNDLRSLFLAERDQVETLASCSK	APKVVILSKALEYLQATVGA		
N-Myc	SERRRNHNILERQRRNDLRSSFLTRDHVPELVKNEK	AAKVVILKKATEYVHSLQAE		
C-Myc	NVKRRTHNVLERQRRNELKRSEFALRDQIPELENNEK	APKVVILKKATAYILSVQAE		
E47	RERRMANNARERVRVRDINEAFRELGRCMCQHLKSDKAQTKLLILQQAQVQVILGLEQQ			
E12	KERRVANNARERLRVRDINEAFKEGRCMCQLHLNSEKPTKLLILHQAVSVILNLEQQ			
myogenin	VDERRAATLEKRRRLKKVNEAFEAALKRSTLLNP	NQR	LPKVEILRSATQYIERLQAL	
Myf5	MDRKAATMRERRRLKKVNOAFETLKRCTTNP	NQR	LPKVEILRNARIRYIESLQEL	
MyoD	ADRRKAATMRERRRLSKVNEAFETLKRCTSSNP	NQR	LPKVEILRNARIRYIEGLQAL	
Twist-DR	SNQRVMANVRERQRTQSLNDAFKSLQQITPTLP	SDK	LSKIQTLLKLATRYIDFLCRM	
Tal1	VVRRIFTNSRERWRQONVNGAFAEERKLIPHTP	PDKKL	SKNETLRLAMKYINFLAKL	
Emc-DR	GRIQRHPTRHGDGENAEMKMYLSKLDLVFPMPKNRK	ETKLEITQHVIDYICDLQTE		
Id1	RLPALLDEQQVNVLLYDMNGCYSRLKELVPTLPQNRK	VSKVEILQHV	VIDYIRDLQLE	
Id2	ISRSKTPVDDPMSLEYNMDCYSRLKELVPSIPQNK	VSKMEILQHV	VIDYILDQLA	
Id3	GRGKGPAEEPLSLDDMNHCSRLRELVPQVPRGTQ	LSQVEILQHV	VIDYILDQVQ	
Id4	KAAEAADEPALCLOCDMDCYSRLRLVPTLPPNKK	VSKVEILQHV	VIDYILDQLA	
DIP1	EVLSTPTQSPENNDLISYNSVWVACQMPQIPRDNK	AAALLMLTKNVDFVKAHEE		

FIG. 2. Multiple sequence alignments of DIP1 with other HLH proteins, within their HLH domain. The positions of the conserved basic region, amphipathic helices 1 and 2, and the loop are indicated [14]. Amino acids conserved among most of the HLH proteins by the canonical rule are shaded.

(about 43 kDa) was also ubiquitously expressed in various cell types (Fig. 4b). The size of this protein is consistent with its predicted size based on the *dip1* cDNA sequence. A larger protein (about 58 kDa) was also seen with the 5C9 antibody in most of these cell lines but its significance is also not known. A Southern "zoo blot" assay suggested that the *dip1* gene is relatively conserved in mammalian species (data not shown). These properties of *dip1* suggest that this gene plays an essential function in several tissues and species.

Effects of the Cell Cycle on DIP1 Expression and Phosphorylation

To examine whether the expression of DIP1 varies during different phases of the cell cycle, HSF8 primary human skin fibroblasts were arrested in G1 by serum starvation and then refed with medium containing 10% fetal bovine serum. Flow cytometry (Fig. 5, bottom) indicated that following the serum starvation (time 0) 94% of the cells were in G1. After refeeding with serum the cells progressed through G1, entered S phase at about 24 h, and reached G2/M at about 30 h. Western blots of extracts of these cells indicated that, as expected the expression of cyclin D1 was induced at about 12–15 h, i.e., during mid-late G1 (Fig. 5, middle). On the other hand, the level of the DIP1 protein was relatively high at time 0 and remained at about the same level throughout the above time course (Fig. 5, top). Similar results were obtained using Rat6 embryo fibroblasts (data not shown).

These results did not exclude the possibility that posttranslational modification of DIP1 might occur during a specific phase(s) of the cell cycle, thus altering its activity during the cell cycle. We found that when a purified GST-DIP1 fusion protein produced in *Escherichia coli* was incubated *in vitro* with [γ - 32 P]ATP and a crude protein lysate from exponentially growing

HCE7 human esophageal cancer cells, this fusion protein was phosphorylated, but the GST control protein was not phosphorylated (Fig. 6a). To examine whether the kinase activity in these cell extracts is cell cycle regulated, Rat6 cyclin D1-overexpressing cells (R6/D1) and vector control (R6/pl) cells [24] were synchronized by serum starvation and refeeding, as described in the legend to Fig. 6. Protein lysates were then collected at different time points after the addition of serum. Two micrograms of GST-DIP1 bound to glutathione-agarose beads were incubated with 200 μ g of the protein lysate from each time point; the beads were then washed and incubated in the kinase reaction buffer in the presence of [γ - 32 P]ATP (Fig. 6b). With extracts from the R6pl cells, the extent of phosphorylation of GST-DIP1 was low at time 0; increased somewhat at 3, 6, and 9 h; and increased markedly at 15 h (Fig. 6b). The latter time point corresponds to the late G1 phase (unpublished data). A previous study from our laboratory demonstrated that overexpression of cyclin D1 in Rat6 cells shortened the G1 phase when compared to R6pl vector control cells [24]. Therefore, parallel kinase assays were performed with extracts of serum-starved and refed R6/D1 cells. The extent of phosphorylation of GST-DIP1 at time 0 was higher than in the R6pl cells (Fig. 6b). Furthermore, a marked increase in the extent of phosphorylation was seen at 9 h, i.e., 6 h earlier than with the R6pl cells. These results are consistent with the shortened G1 and accelerated onset of the S phase seen in the R6/D1 cells [24].

Although a cyclin D1/Cdk4 kinase assay showed that GST-DIP1 was not the substrate (data not shown), our results suggest the possibility that phosphorylation of DIP1 may play a role in the entry of cells into the S phase. We should stress, however, that it remains to be determined whether a similar cell-cycle-dependent phosphorylation of DIP1 also occurs *in vivo*.

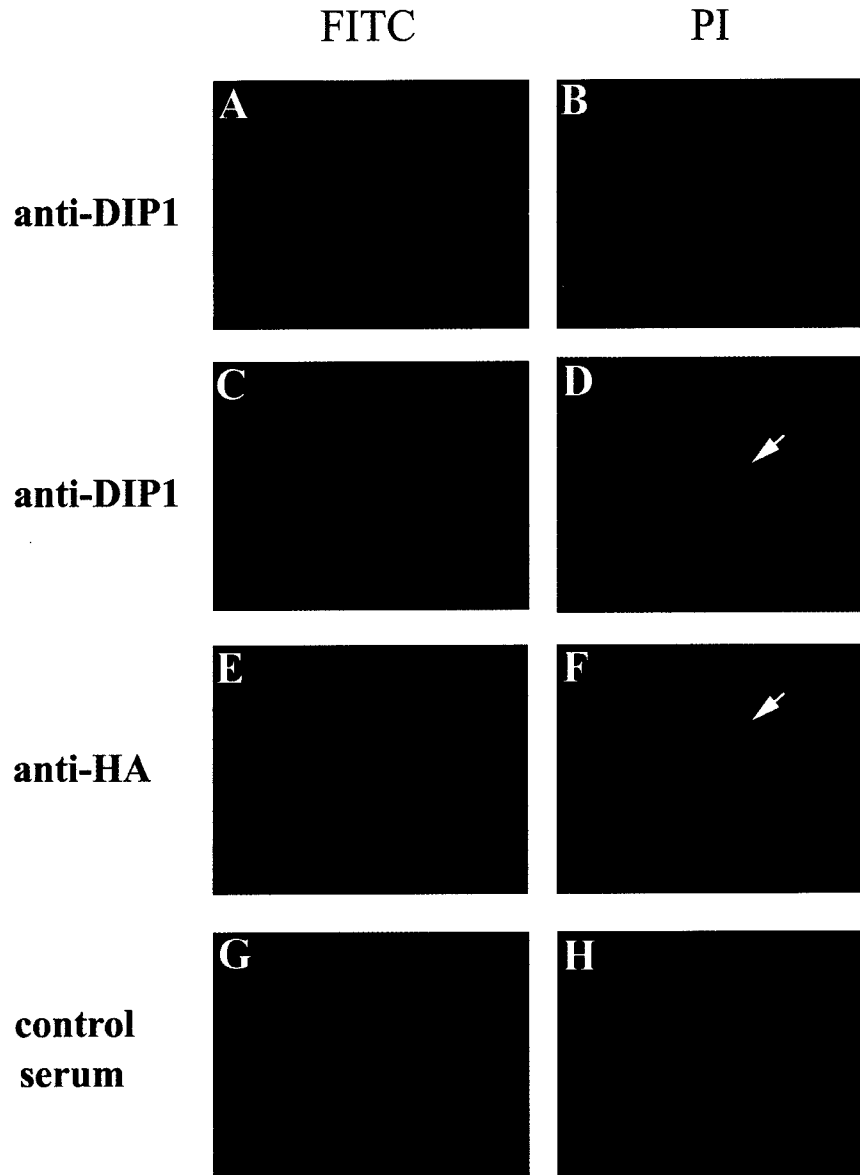


FIG. 3. Nuclear localization of the DIP1 protein by immunofluorescent staining. Green FITC signals (A, C, E, and G) show the DIP1 protein localization and red propidium iodide (PI) signals (B, D, F, and H) show the location of the cell nuclei. Endogenous DIP1 was detected as a nuclear protein in HCE7 cells using the 5C9 anti-DIP1 antibody (A). Exogenous HA-tagged DIP1 was also detected as a nuclear protein in Cos7 cells using the same antibody (C) or the anti-HA 12CA5 antibody (E). As a negative control for C and E, mouse preimmune serum was used instead of the above antibodies (G).

Increased Expression of DIP1 in HL60 Cells during Chemically Induced Differentiation

To investigate the possible involvement of DIP1 in cell differentiation, we have chosen the human promyelocytic leukemia cell line HL60, since it is a very useful model system for studying the effects of various genes and pharmacological agents on differentiation [28]. Treatment of these cells with TPA induces terminal differentiation in the macrophage/monocytic lineages [29], while treatment with retinoic acid or DMSO

results in differentiation in the granulocytic lineage [30]. Western blot analyses were used to examine the pattern of expression of the DIP1 protein during the differentiation of HL60 cells induced by TPA or DMSO. Protein lysates were prepared at different time points after the addition of TPA or DMSO and subjected to Western blot analyses with antibodies specific to pRb, DIP1, or actin (as a control for equal loading) (Fig. 7a). Previous studies indicated that after treatment with TPA or DMSO the cells arrest in G1 at about 24 h and

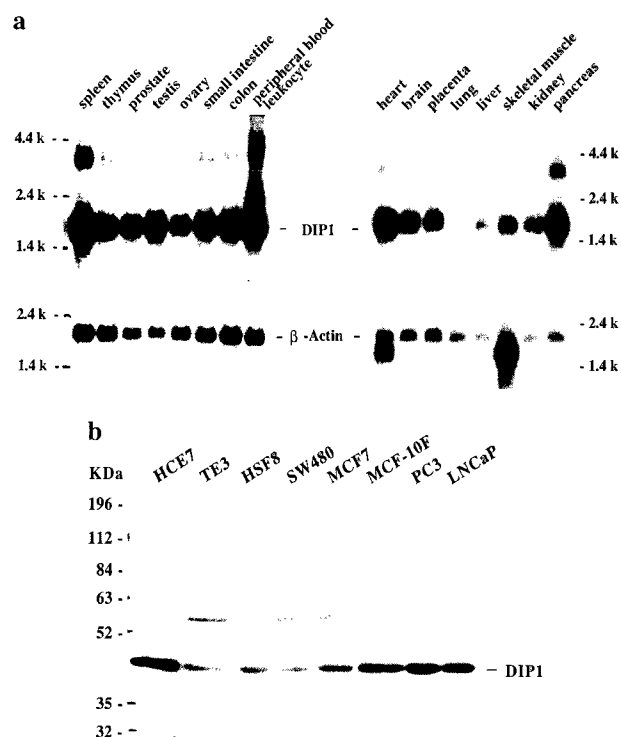


FIG. 4. Ubiquitous expression of DIP1. (a) Expression of the DIP1 mRNA in different human tissues. Northern blot (Clontech) analyses were performed using 32 P-labeled *dip1* cDNA (top) and human β -actin cDNA (bottom) probes. (b) Expression of the DIP1 protein in representative cell lines. Western blot analyses of DIP1 protein expression in different human cell lines were performed using the 5C9 antibody. Fifty micrograms of the protein lysate from each cell line was applied to each lane and analyzed. The original sources of these cell lines were HCE7 and TE3, human esophageal cancer cells; HSF8, primary human skin fibroblasts; SW480, human colon cancer cells; MCF-7, human breast cancer cells; MCF-10F, human normal breast epithelial cells; PC-3 and LNCaP, human prostate cancer cells.

that this is followed by the onset of terminal differentiation [28]. We found that during the differentiation induced by either TPA or DMSO, the phosphorylation status of the retinoblastoma protein pRb changed progressively from the hyperphosphorylated form to the hypophosphorylated form (Fig. 7a, top), which is consistent with a previous study of HL60 cells [31]. We also found a marked increase in the levels of the DIP1 protein at 12 or 18 h, in response to TPA or DMSO, respectively, and these levels continued to increase thereafter, to sixfold at 72 h with TPA and fourfold at 48 h with DMSO (Fig. 7a, middle). The initiation of DIP1 protein induction by TPA and DMSO were both "early events" since they occurred during the first division cycle and prior to the time of full differentiation of the treated cells [32, 33]. Northern blot analyses showed that TPA induced a threefold increase in the level of DIP1 mRNA as early as 6 h after treatment

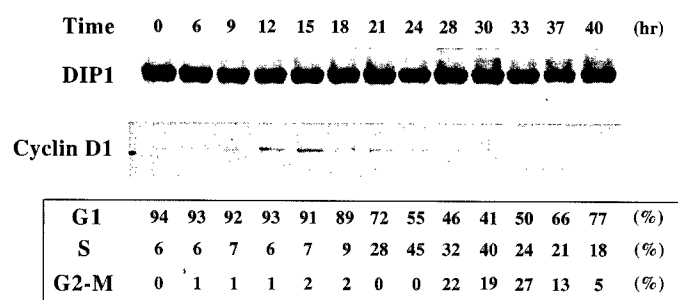


FIG. 5. Constitutive expression of the DIP1 protein throughout the cell cycle. HSF8 cells were synchronized by serum starvation and then refed with serum at time 0. At the indicated subsequent time points after the readdition of serum, protein lysates were collected and Western blot analyses were performed, using antibodies specific for DIP1 (5C9, top) and cyclin D1 (middle). Flow cytometry was also performed on samples of these cells at the same time points and the percentage of cells in each phase of the cell cycle at these time points is indicated (bottom).

(Fig. 7b, left). However, the level of DIP1 mRNA was not significantly increased by DMSO treatment (Fig. 7b, right), indicating that the induction of DIP1 by DMSO is largely a posttranscriptional event.

Chromosomal Localization of the *dip1* Gene

FISH assays were performed to determine the chromosomal location of the *dip1* gene. A human *dip1*

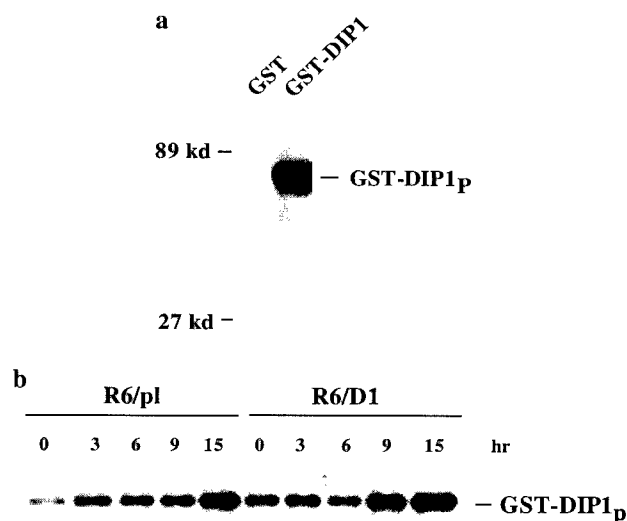


FIG. 6. Cell-cycle-dependent phosphorylation of the GST-DIP1 fusion protein. (a) Phosphorylation of DIP1 by a cellular kinase. Two micrograms of the GST-DIP1 or GST protein was incubated with 100 μ g of a HCE7 cell lysate plus $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the extent of phosphorylation was then assayed, as described under Materials and Methods. (b) R6/pl vector control cells and R6/D1 cyclin D1-overexpressing cells were serum starved and at time 0 stimulated to progress through the cell cycle by adding serum. Extracts were prepared at the indicated time points and assayed for *in vitro* phosphorylation of GST-DIP1, as in (a). Late G1 occurred at about 15 h in R6/pl and about 9 h in R6/D1 cells (unpublished data).

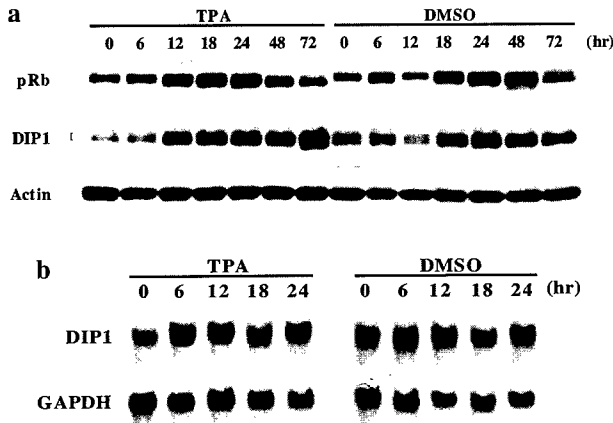


FIG. 7. Increased expression of DIP1 in HL60 cells during differentiation. (a) Western blot analysis of the induction of DIP1 protein by TPA or DMSO. HL60 cells were treated with TPA or DMSO, as described under Materials and Methods. Protein lysates were prepared at the indicated time points and 50 μ g of each sample was subjected to SDS-PAGE and Western blot analyses were performed using an anti-pRb antibody (top) or the anti-DIP1 antibody 5C9 (bottom). Equal loading of each lane was confirmed by probing the same membrane with an antibody specific for actin (bottom). (b) Northern blot analysis of the induction of DIP1 mRNA by TPA or DMSO. HL60 cells were treated with TPA (left) or DMSO (right) for the indicated times and total RNA samples were prepared, as described under Materials and Methods. Northern blots were performed using 32 P-labeled DIP1 (top) or GAPDH (bottom) cDNA as the probes.

genomic clone of about 12 kb was obtained by screening a human λ phage library with a 32 P-labeled *dip1* cDNA probe. This *dip1* genomic clone was subsequently used as the probe in FISH assays with metaphase spreads of normal human lymphocytes. In 21 of 25 (84%) metaphase spreads, specific labeling was seen on one or both chromatids of chromosome 16p11.2-12 (Fig. 8). Similar results were obtained using a different *dip1* genomic clone (data not shown). These results assign *dip1* to a chromosomal region with proximity to genes encoding cyclin F, CREBBP, myosin (MYH11), and tuberous sclerosis-2 (TSC-2). It is of interest that this region of 16p is amplified in a wide range of human cancers [18]. Amplification of 16p has been seen in over 33% of primary human breast cancers [34], and amplification and overexpression of cyclin D1 are also frequently seen in breast cancers [35-37]. These results suggest a possible involvement of DIP1 in the pathogenesis of these cancers.

DISCUSSION

In this paper we describe the identification of a novel gene *dip1* that encodes an Id-like protein (Figs. 1 and 2) that is located mainly in the nucleus of cells (Fig. 3). We found that although DIP1 is expressed in a variety of cell types and its level in fibroblasts does not vary

significantly during the cell cycle, the induction of macrophage-like differentiation by TPA in HL60 cells, and also the induction of granulocyte differentiation by DMSO, was associated with a severalfold increase in cellular levels of this protein. The latter findings suggest that DIP1 may play a role in the differentiation of specific cell types, but this remains to be determined.

In vitro cyclin D1/Cdk kinase assays using a GST-DIP1 recombinant fusion protein as the substrate (instead of pRb) showed that DIP1 is not a substrate of this kinase (data not shown). However, a GST-DIP1 fusion protein was phosphorylated *in vitro* by another cellular kinase(s) present in total protein lysates obtained from HCE7 (Fig. 6a) and Rat6 cells (Fig. 6b). This kinase activity was maximal in extracts from Rat6 fibroblasts that were in the late G1 or early S phase of the cell cycle. Further studies are required to determine whether cell-cycle-specific phosphorylation of DIP1 occurs *in vivo* and the precise kinase(s) involved.

Id proteins are expressed in a variety of cell types [38-43] and often function as negative regulators of cell differentiation. The expression of Id genes is, therefore, down-regulated during differentiation in several cell types [16, 17, 44-47], and ectopic expression of Id proteins can inhibit development and differentiation [38-53]. On the other hand, Id expression is induced during development and differentiation in other types of cells, and the enforced increased expression of Id genes can in some cases induce differentia-

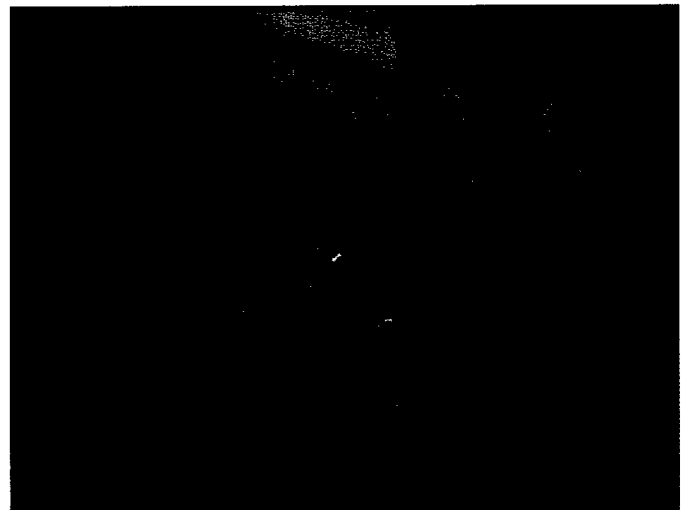


FIG. 8. Chromosomal localization of the *dip1* gene. Fluorescence *in situ* hybridization (FISH) was performed using metaphase spreads of normal human lymphocytes and a human genomic *dip1* λ phage clone as the probe, as described under Materials and Methods. The arrows indicate the blue signals on the regions corresponding to the two chromosome 16-11.2-12 loci. Eighty-four percent of the mitotic spreads analyzed gave similar results, and this finding was confirmed using another *dip1* genomic clone as the probe (data not shown).

tion [54–60]. Furthermore, Id₂ mRNA levels markedly increase during the differentiation of myeloblast cell lines (HL60, PLB-985, THP-1, and U-937) into either granulocytes or macrophages [61]. The latter studies suggest that in some cases Id proteins can antagonize the action of bHLH proteins that suppress the differentiation of specific cell types. Indeed, some bHLH proteins inhibit, rather than enhance, differentiation [62–65]. Through analogous mechanisms, the increases in DIP1 protein that we found in TPA- or DMSO-treated HL60 cells might enhance their differentiation. Studies are in progress to examine this hypothesis.

We should emphasize that although DIP1 was originally identified in a yeast two-hybrid system in which cyclin D1 was used as the “bait,” thus far we have not been able to obtain definitive evidence from coimmunoprecipitation assays that the endogenous DIP1 protein binds to cyclin D1 in mammalian cells (data not shown). Therefore, the precise function of this novel protein remains to be determined. In recent unpublished studies we found that stable overexpression of DIP in MCF7 human breast cancer cells markedly inhibits growth. We are now using this system to analyze the mechanism of action of DIP1.

During the course of this study, Hwang *et al.* reported a novel maternal Id-like protein, designated Maid, that was present in two-cell-stage mouse embryos [27]. They found that the Maid protein inhibited E12/MyoD activities [27], and we have seen similar effects with DIP1 (data not shown), which is consistent with their putative Id-like functions. Maid shares an overall amino acid sequence identity of 78% with DIP1 but lacks the first 50 residues at the N-terminus of DIP1 (data not shown). Therefore, Maid might be the mouse homologue of DIP1. However, the *maid* gene is located on mouse chromosome 2, in a region that is syntenic to human chromosome 15q or chromosome 20, and we found that *dip1* is located on human chromosome 16p11.2-12 (Fig. 8). These findings suggest that DIP1 and Maid might be different members of the same family rather than the same protein in different species. In fact, in FISH assays with a *dip1* genomic fragment as the probe, a weak signal was reproducibly detected on human chromosome 20, in addition to the strong signal at 16p11.2-12 (data not shown). Furthermore, Northern blot analysis with a *dip1* cDNA probe exhibited, in addition to the major 1.6-kb RNA band, a weak 4-kb band (Fig. 4a). These findings suggest that *dip1* belongs to a multigene family, but as emphasized above, the precise functions of DIP1 and Maid remain to be determined.

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Relevance of Dip1, a novel cyclin D1 binding protein, to breast cancer. T. Su, J.Q. Luo, Y. Yao and I. B. Weinstein *Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY 10032.*

There is increasing evidence that in addition to its role in controlling the G1 to S transition of the cell cycle, by complexing with and activating CDK4 and CDK6, cyclin D1 plays a role in other pathways. Using the yeast two-hybrid system we cloned a novel human gene, Dip1, which encodes a 45 kDa nuclear protein that binds to cyclin D1. In this study we found by western blot analysis that Dip1 is expressed at a relatively high level in MCF10F normal human mammary epithelial cells, and at a lower level in both the MCF7 and T47D human breast cancer cell lines. It is also expressed in a series of primary human breast cancers. Co-immunoprecipitation studies indicated that in MCF7 cells, some of the Dip1 protein exists in a multi-protein complex together with cyclin D1 and the estrogen receptor (ER). Assays for ER activity indicated that Dip 1 inhibited the transcription of an estrogen response element (ERE)-luciferase reporter in the presence of cyclin D1 and 17 β -estradiol (E2). We have used an EBV-based vector (pCEP4) to stably overexpress the Dip1 cDNA in MCF7 cells and found that this caused growth inhibition. Further studies are in progress to examine the role of Dip1 in the control of gene transcription in human breast cancer.

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